Results in Immunology 2 (2012) 158–165

Contents lists available at SciVerse ScienceDirect

Results in Immunology

journal homepage: www.elsevier.com/locate/rinim

# Relevance of nuclear receptor expression in a Tchreg cell line, HOZOT: RXRα and PPARγ negatively regulate IFN-γ production

Motoyuki Suzuki[a,*], Makoto Takeuchi[a], Kazue Tsuji-Takayama[a], Akira Harashima[a], Takeshi Otani[a], Terumasa Toraya[a], Hiroki Kakuta[b], Fumiyuki Yamasaki[c], Shuji Nakamura[a], Masayoshi Kibata[a]

[a]Cell Biology Institute, Research Center, Hayashibara Biochemical Laboratories, Inc., 675-1 Fujisaki, Nakaku, Okayama 702-8006, Japan
[b]Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Tsushima-Naka, Okayama, Japan
[c]Kurashiki Medical Center, Bakuro-cho, Kurashiki, Okayama, Japan

ARTICLE INFO

*Article history:*
Received 25 June 2012
Received in revised form 8 August 2012
Accepted 13 August 2012

*Keywords:*
RXRα
PPARγ
IFN-γ
HOZOT

ABSTRACT

Nuclear receptors (NRs) have recently received much attention for their newly discovered roles in T cell development, as exemplified by RARα (Treg cells) and RORγt (Th17 cells). In previous studies, we characterized a new type of T cell subset, designated as Tchreg (cytotoxic, helper, and regulatory T) cells, in terms of its cytokine signature. In this study, we investigated the expression and functional relevance of NRs in Tchreg cells by performing mRNA profiling of HOZOT, a cord blood-derived Tchreg cell line. We identified eleven inducible and eight constitutively expressed NRs in HOZOT. Among these NRs, RXRα and PPARγ showed features of signature NRs of Tchreg cells because they were selectively expressed in HOZOT compared with other T cell subsets. These NRs exhibited contrasting expression patterns, as RXRα was independent of anti-CD3/28 antibody stimulation while PPARγ was stimulated-dependent. Upon agonist treatment, both proteins translocated to the nucleus and inhibited IFN-γ production through binding to the promoter region of the IFN-γ gene. These results provide new insight into the roles of RXRα and PPARγ in T cell biology, especially in their biological relevance in Tchreg cells.



## 1. Introduction

Nuclear receptors (NRs) are ligand-activated transcription factors that modulate gene expression through binding to specific hormone response elements. A total of forty-eight human NRs have been identified and classified into seven groups: the thyroid hormone receptor family, the retinoid X receptor family, the estrogen receptor family, the neuron growth factor IB family, the steroid family, the germ cell nuclear factor, and others [1]. Since NRs function as transcription factors, their roles include diverse physiological and pathological processes such as cellular development and differentiation, metabolic homeostasis, cancer, autoimmune diseases, inflammatory diseases, and diabetes [2,3].

Studies of NRs have recently focused on T cell biology. Naïve $CD4^+$ T cells differentiate into distinct types of T cells under the appropriate inducing conditions. Among them, T helper (Th) 17, a subset of T helper cells characterized by secretion of Interleukin (IL)-17, have been associated with the pathogenesis of autoimmunity [4–6] and their development was linked to the expression of retinoic acid-related orphan receptor (ROR) γt [7]. Under Th17 differentiation conditions, IL-6 and transforming growth factor (TGF)-β induce the expression of RORγt, which directly or indirectly promotes IL-17 transcription. However, it is not yet known whether this process is ligand-dependent. Regulatory T (Treg) cells are another example of NR-controlled T cells. Forkhead-winged helix family transcription factor $3^+$ Treg cells are induced in a manner reciprocal to Th17 cells by IL-2 and TGF-β, and a retinoic acid receptor (RAR) ligand such as retinoic acid can enhance their differentiation [8]. Tr1 cells (an IL-10-producing type of Treg cell), are induced by an active form of vitamin D3 [9]. Interestingly, retinoic acid promotes Treg cell differentiation in the intestine whereas vitamin D3 does the same in the skin, highlighting specialized roles for nuclear receptor ligands in local tissues.

In previous studies, we described a new and distinct type of Treg cell line, termed HOZOT [10]. HOZOT exhibited multifunctional properties such as suppression of mixed lymphocyte reaction (MLR), helper activity under anti-CD3 stimulation conditions, cytotoxic activity and cell-in-cell activity against human tumor cells [10–13]. Therefore, we designated these cells as Tchreg (cytotoxic, helper, and regulatory) cells. By mRNA profiling, cytokines and chemokines such as IFN-γ, IL-10, RANTES, and IL-8 were identified as signature molecules of Tchreg cells [14,15]. We also reported the low expression of micro RNA (miR)-155 as a characteristic of Tchreg cells, in contrast to the high miR-155 expression levels observed in natural Treg cells [16]. Since NRs play important roles in T cell development and function, we focused this study on the biological relevance of NR

* Corresponding author. Tel.: +81 86 276 8621; fax: +81 86 274 2150.
*E-mail address:* motoyuki.suzuki@hb.nagase.co.jp (M. Suzuki).

2211-2839 
http://dx.doi.org/10.1016/j.rinim.2012.08.001

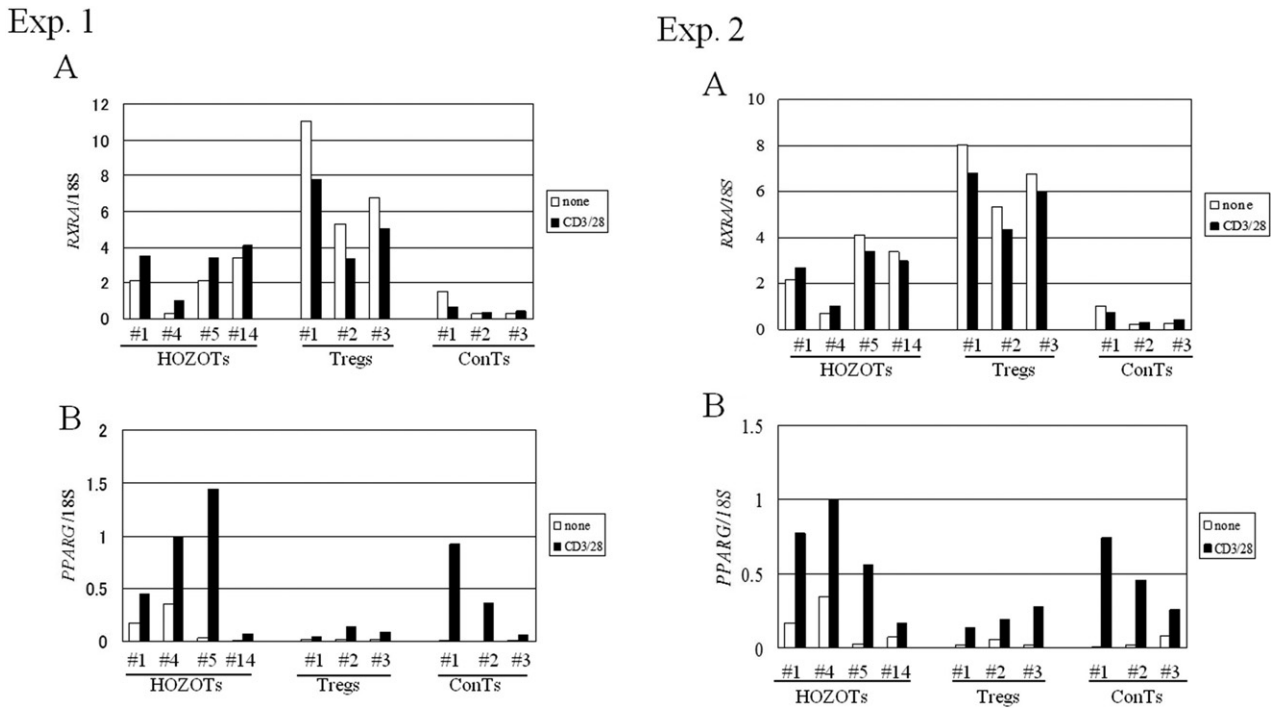


**Fig. 1.** *RXRA* and *PPARG* were defined as signature NRs by comparing their expression in HOZOTs to those in other T cell subsets. Expression of *RXRA* (A) or *PPARG* (B) mRNA in T cell subsets is shown as a ratio of *RXRA* or *PPARG* mRNA to 18 S rRNA, respectively. RNA was harvested after 24 h culture with or without anti-CD3/28 antibody stimulation, and the relative amount of mRNA was measured by RT-qPCR. All values were standardized by defining the sample value of the anti-CD3/28 antibody stimulated HOZOT (#4) as 1.0. Results of two independent experiments are presented as experiment 1 (left) and 2 (right).

expression and function in Tchreg cells. To accomplish this, we first examined mRNA expression of NRs in HOZOT using DNA microarray analysis and identified retinoid X receptor$\alpha$ (RXR$\alpha$) and peroxisome proliferator-activated receptor (PPAR)$\gamma$ as signature NRs. We further investigated the effects of agonistic ligands of these NRs on protein localization and cytokine production.

## 2. Materials and methods

### 2.1. Reagents

Interleukin (IL)-2 was purchased from Pepro Tech EC Ltd. (London, UK). Anti-CD3$\varepsilon$ (UCHT-1), anti-CD28 (37407) antibodies, and RANTES ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). IL-10 ELISA kits were purchased from eBioscience (San Diego, CA, USA). IFN-$\gamma$ ELISA kit was obtained from Hayashibara Biochemical Labs. (Okayama, Japan). Anti-PPAR$\gamma$ (E-8), either Alexa 488-conjugated or unconjugated, and anti-human RXR$\alpha$ (D-20) antibodies were purchased from Santa Cruz Biosciences (Santa Cruz, CA, USA). Ligands for NRs were obtained as follows: thiazolidinedione (TZD) and GW9662 were purchased from WAKO chemical (Osaka, Japan) and Calbiochem (San Diego, CA), respectively. NEt-3IP (RXR specific agonist) and NS-4TF (RXR antagonist) were synthesized as described in the previous reports [17–19].

### 2.2. Generation of HOZOT cells

The generation of HOZOT cell lines was described in detail previously [10]. Briefly, umbilical cord blood (UCB) samples were collected at Kurashiki Medical Center after obtaining informed consent according to the Declaration of Helsinki. Mononuclear cells (MNC) derived from UCB were enriched using Ficoll-paque density centrifugation and co-cultured with mouse stromal cell lines, ST2 or MS-5, in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Two to three weeks later, proliferative cells, which exhibited cytotoxicity against the stromal cell line, were expanded in the presence of IL-2 (10 ng/mL). The expanded cells, termed HOZOT, were used for further analysis.

### 2.3. Cell preparation

The preparation of conventional T (ConT) cells and naturally occurring T regulatory (nTreg) cells was described previously. UCB-derived $CD25^+$ cells were isolated by positive selection with directly conjugated anti-CD25 magnetic beads (Militenyi Biotech, Bergisch Gladbach, Germany). The purity of $CD25^+$ cells were >90%. $CD25^+$ T cells were used for the preparation of nTreg cells. $CD25^-$ cells were treated with anti-CD4 magnetic beads (Militenyi Biotech), and $CD4^+$ cells were enriched using the Midi-MACS system. The purity of $CD4^+CD25^-$ cells were >95%. $CD4^+CD25^-$ T cells were used for the preparation of ConT cells. $CD25^+$ T cells and $CD4^+CD25^-$ T cells were stimulated on plates coated with anti-CD3/CD28 antibodies in RPMI-1640 medium containing 10% FBS at 37 °C in 5% $CO_2$ in the presence of 10 ng/mL IL-2. Growing cells were used as nTreg and ConT cells.

### 2.4. DNA microarray analysis

Preparation of DNA microarray samples was described previously [15]. Total RNA from HOZOT was isolated with RNeasy kit (Quiagen, Valencia, USA) according to the manufacturer's instructions. The samples were processed and analyzed at the Bio Matrix Research Institute (Nagareyama, Chiba, Japan) using U133 Plus 2.0 Affymetrix Gene Chip arrays containing 54,675 human genes and transcripts. DNA microarray data of HOZOT were normalized and analyzed by Gene Spring GX software (Agilent Technologies, Wilmington, DE, USA).

### 2.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

The expression of NRs was analyzed at the mRNA level by RT-qPCR. Total RNA was isolated from HOZOT, ConT cells, nTreg cells and $CD4^+CD25^-$ T cells using RNeasy kit (Qiagen). RT-qPCR was done as

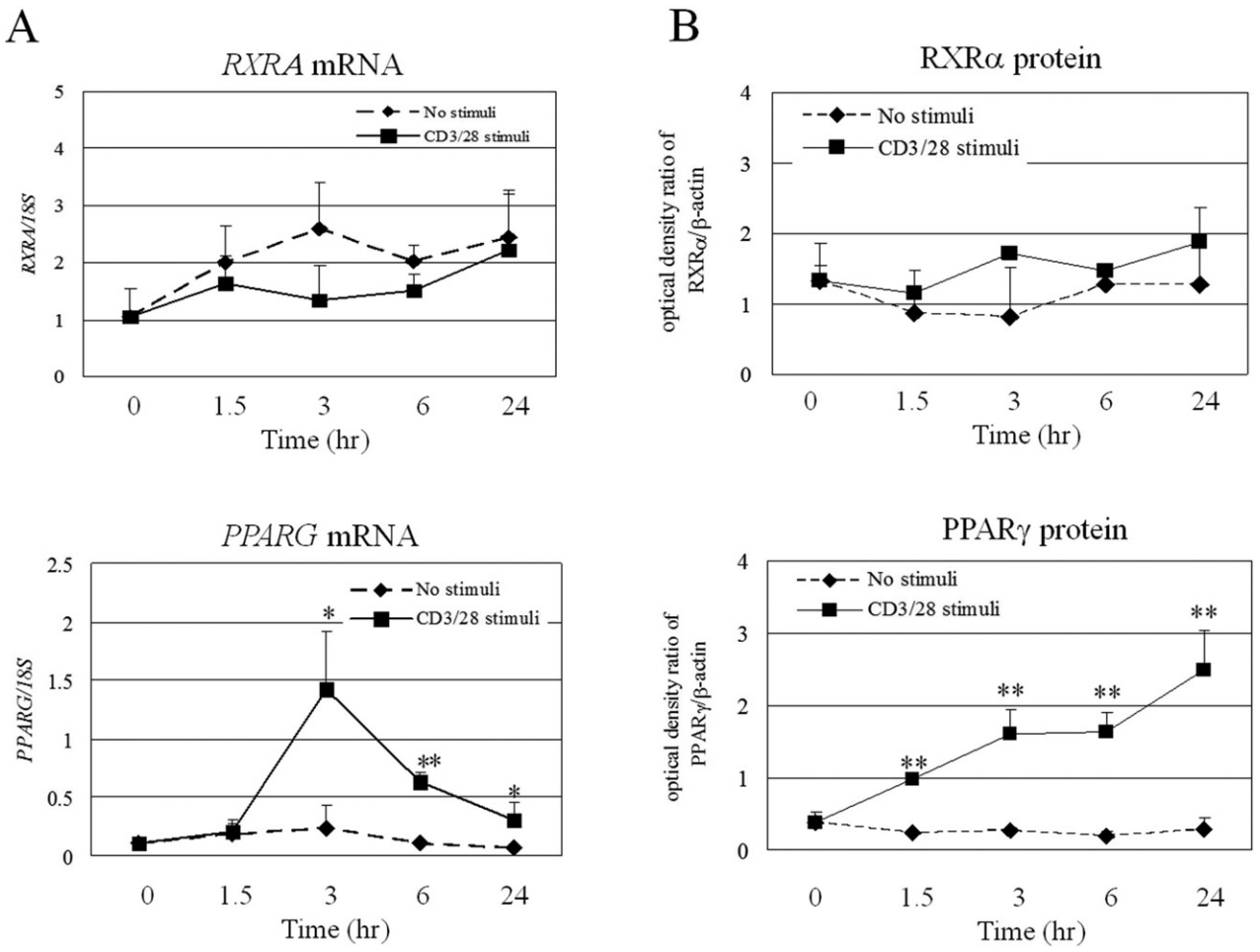


**Fig. 2.** Time course study showed *RXRA* as a constitutively expressed gene and *PPARG* as an inducible gene. (A) The time course of *RXRA* or *PPARG* mRNA expression by HOZOT cells was analyzed. The amount of *RXRA* or *PPARG* mRNA in 0, 1.5, 3, 6, and 24 h culture samples was measured by RT-qPCR. The ratios of *RXRA* or *PPARG* mRNA to 18 S rRNA are shown after standardization as in Fig. 1. (B) The time course of RXRα or PPARγ protein expression was studied. RXRα or PPARγ protein in 0, 1.5, 3, 6, and 24 h culture samples was detected by Western blotting using anti-RXRα or anti-PPARγ antibody. After stripping, the membrane was reprobed with anti-β-actin antibody. The optical density ratios of RXRα or PPARγ bands relative to a corresponding β-actin band are shown for quantitative analysis. The values are shown as the means ± SD of three experiments. * and **; $p<0.05$ and $p<0.01$ compared with the values of the control without antibody stimulation, respectively.

described previously [15]. The primer sequences used in this study are described elsewhere (Suppl. Fig. 1).

### 2.6. Immunohistochemistry

Cells were cytospun onto slide glasses and fixed with ice-cold methanol for 2 min. After treatment with blocking buffer containing 1% FBS in PBS, RXRα proteins were stained using an anti-RXRα antibody and Alexa 488-conjugated anti-rabbit IgG antibody, and PPARγ was stained with Alexa 488-conjugated anti-PPARγ antibody. The nuclei were counterstained with 0.5 μg/mL Hoechst 33258 in PBS.

### 2.7. Western blotting analysis

Western blotting was performed as previously described [20]. Briefly, equivalent amounts of total protein were loaded onto 10% SDS polyacrylamide gels. After electrophoresis, proteins were electrotransferred onto the nitrocellulose membranes and reacted with the appropriate primary antibody according to standard methods. Bound immunocomplexes were visualized by use of Super Signal West Pico (Pierce, Rockford, IL). In some experiments, the membranes were stripped and then reprobed with anti-acetyl histone H3 antibody (Millipore International, Inc., Temecula, CA) or anti-β-actin antibody to confirm the equality of total nuclear or cytoplasmic protein loading.

### 2.8. Cytokine production assays

Six-well flat-bottom plates were precoated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) antibodies. HOZOT cells were cultured in the six-well flat-bottom plates at $1 \times 10^6$/well/mL for 16 h, in RPMI-1640 medium containing 10% FBS and 10 ng/mL IL-2. HOZOT cells were collected and washed with RPMI-1640 medium containing 10% FBS and resuspended in the same medium. HOZOT cells were cultured in 24-well flat-bottom plates, which were coated with anti-CD3/CD28 (1 μg/mL) antibody at $5 \times 10^5$/well for one day with or without increasing concentrations of TZD, NEt-3IP, GW9662, and NS-4TF, each alone or in combination. Cell culture supernatants were harvested and analyzed for IFN-γ, RANTES and IL-10 production using ELISA kits.

### 2.9. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation was performed as previously described [14]. Briefly, HOZOT cells were fixed with 1% formaldehyde for 10 min at room temperature, and then the fixation was stopped with 1.25 M glycine. Fixed cells were washed with cold PBS. Cells were treated with lysis buffer (Santa Cruz Biotechnology) and sonicated six times (10 s each) to prepare chromatin extracts. Protein G beads (100 μL) were incubated with anti-PPARγ antibody (5 μg), anti-RXRα antibody (5 μg), control mouse IgG (5 μg), or control rabbit

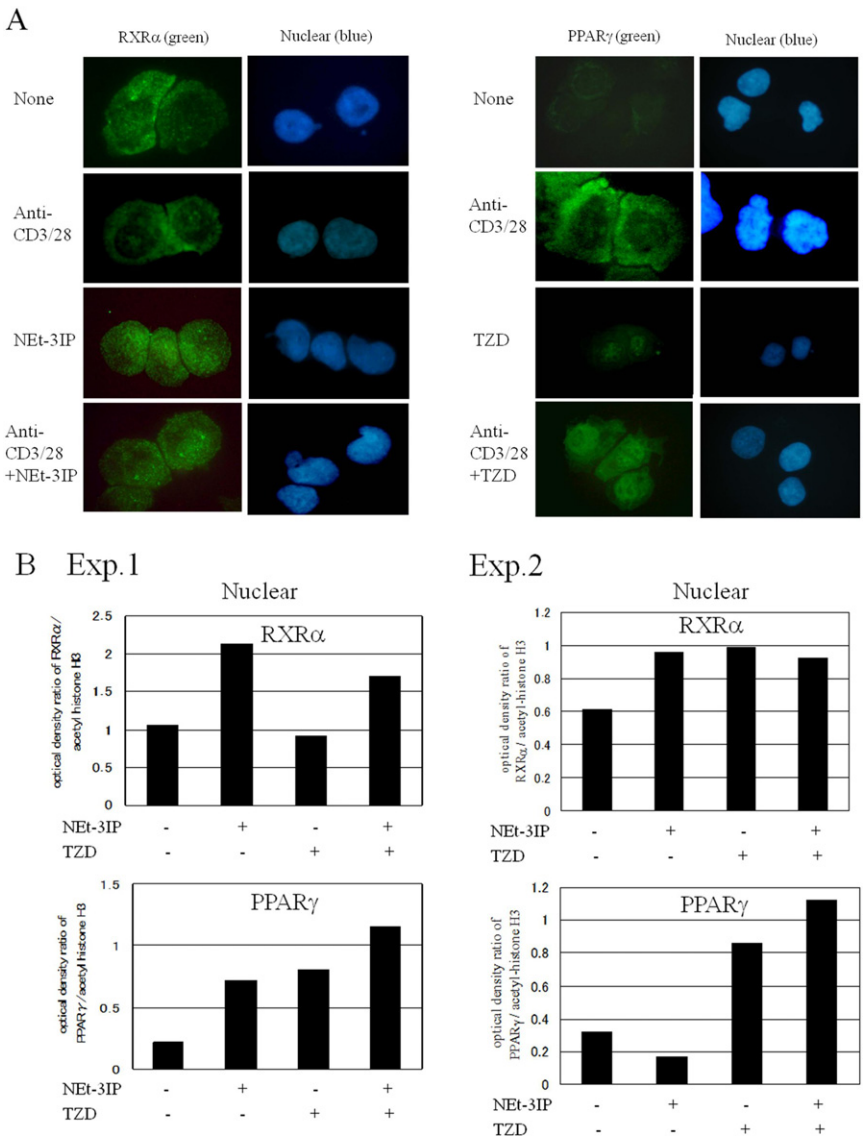


**Fig. 3.** Cellular localization of RXRα or PPARγ protein analyzed by immunohistochemistry and Western blotting. (A) HOZOT cells were stimulated with 1 μg/mL of anti-CD3/28 antibody for 16 h and then treated with 1 μM TZD or NEt-3IP for 3 h. For immunohistochemistry, cells were stained with rabbit anti-RXRα antibody (green) or mouse anti-PPARγ antibody (green) and counterstained with Hoechst 33258 (blue). (B) HOZOT cells were stimulated with 1 μg/mL anti-CD3/28 antibody for 16 h and then treated with 1 μM TZD and/or NEt-3IP for 3 h. Nuclear fraction was prepared and the subcellular localization of RXRα or PPARγ was determined by Western blotting. After stripping, the membrane was reprobed with anti-acetyl-Histone H3 antibody. The optical density ratios of RXRα or PPARγ bands relative to corresponding acetyl-Histone H3 bands are shown for quantitative analysis. Results of two independent experiments are presented as experiment 1 (left) and 2 (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IgG (5 μg) at 4 °C overnight. Protein G beads were washed three times with cold PBS and then chromatin extracts were incubated with Protein G beads at 4 °C overnight. After washing the immunoprecipitates with washing buffer (Santa Cruz Biotechnology), DNA was eluted by incubation with extraction buffer (Santa Cruz Biotechnology) at 67 °C overnight. The DNA samples were purified and used as templates for RT-qPCR. Bioinformatic analysis was performed using rVISTA. The PPARγ response element (PPRE) of the double repeat (DR)1-type was located in the IFN-γ promoter region at 493 bp upstream of the *IFN-G* transcription start site.

### 2.10. Statistical analysis

Statistical analysis was performed by one-way ANOVA, followed by LSD post hoc test, with an acceptable significance probability level at $<0.01$ or $<0.05$.

## 3. Results

### 3.1. Expression profiles of NRs in HOZOT

In our previous studies, we characterized HOZOT by defining the cells' cytokine signature [15] and found that two chemokines, RANTES and IL-8, were produced at high levels by HOZOT.

To further analyze signature molecules, we focused on NR expression in HOZOT by comparing three conditions: unstimulated, ST2-stimulated, and anti-CD3/CD28 antibody-stimulated. The results of DNA microarray analysis are summarized in Table 1. We found that 19 NRs (*RARA*, *PPARA*, *PPARB*, *PPARG*, *REV-ERBAA*, *REV-ERBAB*, *RORA*, *RORG*, *LXRB*, *VDR*, *RXRA*, *RXRB*, *TR2*, *TR4*, *ERRA*, *GR*, *NGFIB*, *NURR1*, and *NOR1*) were expressed in HOZOT-4 among a total of human 48 NRs. All of the expressed genes were categorized into two groups, either inducible or constitutively expressed genes (Table 1). The inducible genes, the expression of which was increased two- to 3347-fold by

**Table 1**
Nineteen nuclear receptors expressed in HOZOT were detected by DNA microarray analysis.

| Common name | | Symbol | Genbank | Fold change | | | Raw data | | | Flags | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (-) | ST2 | CD3/28 | (-) | ST2 | CD3/28 | (-) | ST2 | CD3/28 |
| Inducible | | | | | | | | | | | | |
| | *RARA* | NR1B1 | AV657604 | 1 | 1.7 | 2.1 | 13.0 | 23.0 | 37.6 | P | P | P |
| | *PPARG* | NR1C3 | AK027107 | 1 | 2.3 | 15.9 | 7.1 | 17.0 | 155.8 | A | P | P |
| | *REV-ERBAA* | NR1D1 | X72631 | 1 | 2.6 | 12.9 | 9.7 | 26.6 | 172.2 | A | A | P |
| | *REV-ERBAB* | NR1D2 | AI761621 | 1 | 0.7 | 3.3 | 43.4 | 34.0 | 195.6 | P | P | P |
| | *RORG* | NR1F3 | AI218580 | 1 | 0.9 | 11.7 | 3.3 | 3.2 | 51.7 | A | A | P |
| | *VDR* | NR1I1 | AA772285 | 1 | 2.1 | 8.2 | 132.5 | 296.1 | 1504.2 | P | P | P |
| | *ERRA* | NR3B1 | L38487 | 1 | 1.3 | 2.0 | 71.3 | 95.7 | 196.5 | P | P | P |
| | *GR* | NR3C1 | AI432196 | 1 | 0.9 | 2.2 | 489.4 | 471.5 | 1501.1 | P | P | P |
| | *NGFIB* | NR4A1 | NM_002135 | 1 | 3.9 | 112.3 | 5.4 | 22.5 | 837.0 | A | P | P |
| | *NURR1* | NR4A2 | NM_006186 | 1 | 2.6 | 247.3 | 11.3 | 30.9 | 3856.6 | A | P | P |
| | *NOR1* | NR4A3 | U12767 | 1 | 1.5 | 3346.8 | 0.8 | 1.3 | 3693.9 | A | A | P |
| Constitutive | | | | | | | | | | | | |
| | *LXRB* | NR1H2 | NM_007121 | 1 | 1.1 | 1.7 | 76.3 | 88.4 | 175.9 | P | P | P |
| | *PPARA* | NR1C1 | AF086231 | 1 | 1.7 | 1.4 | 11.5 | 20.2 | 22.9 | P | P | P |
| | *PPARB* | NR1C2 | BC002715 | 1 | 1.0 | 0.9 | 17.2 | 18.2 | 20.3 | P | P | M |
| | *RORA* | NR1F1 | AK025371 | 1 | 0.8 | 0.4 | 31.7 | 25.9 | 17.6 | P | P | P |
| | *RXRA* | NR2B1 | NM_002957 | 1 | 1.3 | 0.7 | 53.6 | 71.2 | 55.3 | P | P | P |
| | *RXRB* | NR2B2 | BC001167 | 1 | 0.7 | 0.9 | 105.3 | 80.1 | 136.7 | P | P | P |
| | *TR2* | NR2C1 | NM_003297 | 1 | 0.9 | 0.6 | 113.1 | 112.1 | 100.2 | P | P | P |
| | *TR4* | NR2C2 | AL138444 | 1 | 1.1 | 0.5 | 346.3 | 394.2 | 228.9 | P | P | P |

anti-CD3/CD28 stimulation, included *RARA*, *PPARG*, *REV-ERBAA*, *REV-ERBAB*, *RORG*, *VDR*, *ERRA*, *GR*, *NGFIB*, *NURR1*, and *NOR1*. The constitutively expressed genes, the expression levels of which were less than two-fold even after antibody stimulation, included *LXRB*, *PPARA*, *PPARB*, *RORA*, *RXRA*, *RXRB*, *TR2*, and *TR4*.

### 3.2. High expression of RXRA and PPARG as candidate HOZOT markers

Next, we compared the expression of the NRs listed in Table 1 among different T cell subsets (HOZOT, nTreg cells, ConT cells, and naïve T cells) using RT-qPCR. HOZOT preferentially expressed five NRs: *PPARG*, *NGFIB*, *NURR1*, *NOR1*, and *RXRA* (data not shown). To validate the expression profiles of these NRs, we examined their expression by RT-qPCR using four sets of HOZOTs and three sets each of ConT and nTreg cells. *RXRA* mRNA was highly expressed in three of the four sets of HOZOTs and all three sets of Tregs cell lines compared to the three sets of ConT cell lines, whereas the mRNA levels of *PPARG* in HOZOTs (except for #14) and ConT cells (except for #3) was higher than in Tregs (Fig. 1). On the other hand, the expression levels of *NGFIB*, *NURR1*, and *NOR1* mRNA varied widely even among the four sets of HOZOTs (data not shown). Therefore, we focused our interests on *RXRA* and *PPARG* to explore the relevance of these NRs in HOZOT's characterization.

### 3.3. Time course of mRNA and protein expression of RXRA and PPARG in HOZOT

As shown above, *RXRA* was constitutively expressed while *PPARG* was inducible. Time course studies revealed in detail the cells' expression at both mRNA and protein levels. Unstimulated HOZOT cells expressed significant levels of RXR$\alpha$ mRNA and protein, whereas they expressed low levels of PPAR$\gamma$ mRNA and protein (Fig. 2A and B). Even after anti-CD3/CD28 antibody stimulation, RXR$\alpha$ expression did not change much between 1.5 and 24 h. In contrast, PPAR$\gamma$ expression increased after antibody stimulation and reached a maximum after 3 h (Fig. 2A). PPARg protein expression increased steadily upto 24 h after antibody stimulation (Fig. 2B). We confirmed these properties by flow cytometric analysis (data not shown).

### 3.4. Subcellular localization of RXR$\alpha$ and PPAR$\gamma$ in HOZOT cells

To examine the subcellular localization of RXR$\alpha$ and PPAR$\gamma$ proteins in HOZOT cells, we performed immunohistochemical analysis and Western blotting analysis using nuclear extracts. RXR$\alpha$ protein was detected in the cytoplasm under both anti-CD3/28 antibody stimulated and unstimulated conditions. On the other hand, PPAR$\gamma$ protein was weakly detected under unstimulated conditions, but upon Ab stimulation, it appeared in the cytoplasm at high levels. When treated with agonistic ligands for RXR$\alpha$ and PPAR$\gamma$, NEt-3IP and TZD, respectively, both RXR$\alpha$ and PPAR$\gamma$ proteins were translocated to the nuclei (Fig. 3A). NEt-3IP is an agonist specific for RXR$\alpha/\beta$ but not

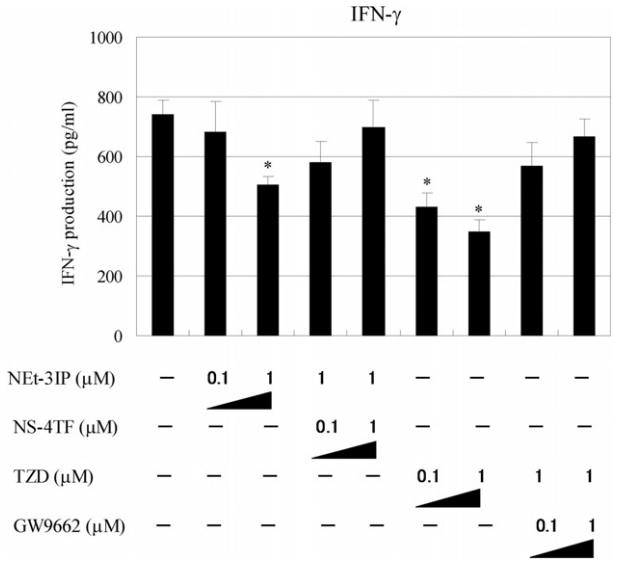


**Fig. 4.** The suppressive effects of agonists or antagonists of RXRα or PPARγ on IFN-γ production by HOZOT cells. IFN-γ production was measured by stimulating HOZOT cells with 1 μg/mL anti-CD3/28 antibody for 16 h. For the agonist and antagonist treatment, HOZOT cells were pretreated for 30min with 0.1 and 1 μM NS-4TF (RXRα antagonist) or GW9662 (PPARγ antagonist) and then treated with 0.1 and 1 μM NEt-3IP or TZD. Culture supernatants were collected for determining IFN-γ levels by ELISA. The values are shown as the means ± SD. Data are representative results of at least two independent experiments. * $p<0.01$ compared with the values of the control without ligand stimulation.


**Fig. 5.** Bioinformatics analysis of the PPRE/DR-1 region in the IFN-γ promoter region. The sequence of the IFN-γ promoter region from – 810bp to 0bp. The PPRE/DR-1 sequence is underlined and marked red. Primers used for Chip-qPCR are marked with black arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RXRγ, whereas TZD is specific for PPARγ but not PPARα/β. Western blotting analysis confirmed the localization of both NRs (Fig. 3B).

### 3.5. NEt-3IP and TZD inhibit IFN-γ production by HOZOT cells

To further investigate the functional relevance of RXRα and PPARγ proteins in HOZOT cells, we treated HOZOT cells with their agonistic ligands and observed the effects on cytokine productions. HOZOT cells produce high amounts of IFN-γ, RANTES, and IL-10. Among them, IFN-γ production was inhibited in a dose-dependent manner by either NEt-3IP or TZD treatment alone (Fig. 4). To examine antagonist effects, we next treated HOZOT cells with combinations of agonists and antagonists, namely RXRα agonist (NEt-3IP) plus antagonist (NS-4TF) or PPARγ agonist (TZD) plus antagonist (GW9662). Each antagonist abolished its corresponding agonist's effects on IFN-γ production (Fig. 4). In contrast, no significant decrease or increase in RANTES and IL-10 production was observed by NEt-3IP and TZD treatment (Suppl. Fig. 2). Cell viability was maintained at high levels at concentrations upto 10 mM for both ligands (data not shown). These results indicated that both NRs were functionally involved in IFN-γ production in HOZOT cells.

### 3.6. PPARγ and RXRα directly bind to the IFN-γ gene

To explain the mechanism of suppression of IFN-γ production by NEt-3IP and TZD in HOZOT cells, we hypothesized that RXRα and PPARγ could directly bind to the IFN-γ promoter region. We first performed a bioinformatics search for DR1 and PPRE sites, specific binding sequences for nuclear receptors, on the IFN-γ promoter region using web-based software, rVISTA. A DR1-type PPRE was found in the IFN-γ promoter region at 493 bp upstream of the *IFN-γ* transcription start site (Fig. 5). We next performed a ChIP analysis with anti-RXRα antibody and anti-PPARγ antibody using HOZOT cells treated with NEt-3IP or TZD. As shown in Fig. 6, low amounts of RXRα and PPARγ proteins were bound to the IFN-γ promoter region, even in ligand-unstimulated HOZOT cells. Upon ligand stimulation, increased amounts of RXRα and PPARγ proteins were detected in Chip samples with specific antibodies but not in those with control antibodies. These results supported our hypothesis that the inhibitory effects of RXRα and PPARγ agonists on IFN-γ production operated through the direct binding of these agonists to the IFN-γ promoter.

## 4. Discussion

Expression profiles of NRs during cell development and differentiation have been reported by a number of investigators. For example, Xie et al. [21] reported that ERRβ, DAX-1 and LRH-1 controlled the development of embryonic stem cells. According to Barish et al. [22] 28 NRs including RXRα and PPARγ are involved in the activation of macrophages. A number of NRs control T cell differentiation, for example RORγt serving as a specific marker of Th17 cells. Thus, comprehensive analysis of NR expression in a new type of T cell, HOZOT, was a necessary and interesting step to enhance our understanding of this cell line. In this study, we found that 19 of 48 NRs were expressed in HOZOT, and identified eight genes (*RXRA* and *TR2*, etc.) that were constitutively expressed, and 11 genes (*NGFIB*, *NOR1*, *NURR1* and *PPARG*, etc) that were inducible. Among them, *RXRA* and *PPARG* were selected for further analyses due to their more selective expression patterns compared with other T cell subsets.

HOZOTs expressed both NRs at relatively high levels, whereas Tregs showed the highest and the lowest expression of RXRα and PPARγ, respectively. It is intriguing that the two NR expression patterns were identified in T cell subsets, suggesting distinct functional roles for these NRs in T cell biology. RXRα functions as a transcription factor by forming a heterodimer complex with other NRs such as PPARγ, VDR, LXRβ, RARα, and NURR1. Therefore, RXRα availability determines the function of these NRs. It is noteworthy that the expression of RXRα remained unchanged after ST2 or T cell receptor stimulation whereas the expression of PPARγ, VDR, RARα, and NURR1 was increased by stimulation. Given the fact that T-lymphocyte proliferation and survival were diminished by RXRα disruption [23] and that the anti-apoptotic gene Bcl2a1 was up-regulated by RXRα agonist treatment [24], RXRα may play a central role by balancing the availability for heterodimer formation with other NR partners. In this regard, it will be interesting to elucidate how HOZOTs control heterodimer complex formation.

Since the *in vivo* counterpart of HOZOTs has not yet been identified, the physiological relevance of high-level expression of RXRα and PPARγ remains unclear. In general, RXR plays important regulatory roles in metabolic disorders, such as type 2 diabetes, hyperlipiderma, and atherosclerosis [25,26] and also in the control of innate inflammatory responses [27]. PPARγ possesses a broad range of biological

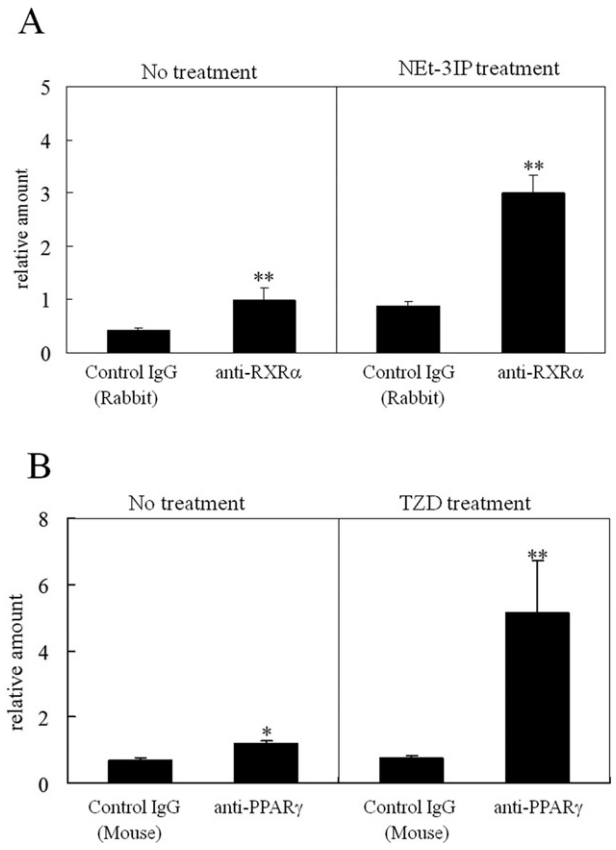


**Fig. 6.** Chromatin immunoprecipitation analysis of RXRα or PPARγ binding to DR1/PPRE. Cross-linked chromatin complexes from NEt-3IP-treated, TZD-treated or non-treated HOZOT cells were immunoprecipitated with anti-RXRα, anti-PPARγ or control IgG. ChIP-qPCR was performed using primers described in Fig. 5. Relative amounts of the PCR product were normalized for those of corresponding input chromatin, and their relative precipitation was expressed as fold precipitation above the baseline. The values are shown as the means ± SD. Data are representative results of three independent experiments. * $p<0.01$ compared with the values of control IgG.

activities in regulating lipid and glucose metabolism, and also negatively modulating inflammation [28,29]. Anti-inflammatory roles of these two NRs have been shown in conditional KO mice. *Rxra* KO mice displayed elevated tumor necrosis factor-α and IFN-γ expression [23], whereas PPARγ KO mice exhibited elevated inducible nitric oxide synthase and IFN-γ expression [29].

By *in vitro* functional studies, we demonstrated that both RXRα and PPARγ agonistic ligands have inhibitory effects on IFN-γ production by HOZOTs. PPARγ agonistic ligands exert inhibitory effects on cytokine production by T cells through various mechanisms, including inhibition of T cell activation, inhibited production of IL-2, induction of apoptosis, inhibition of IL-12 production by antigen presenting cell, or reduced IFN-γ production due to suppressed activation of c-Jun [28,30–32]. In our system, it is interesting that either RXRα or PPARγ ligand independently but not synergistically inhibited IFN-γ production by HOZOTs (data not shown). To the best of our knowledge, we demonstrated for the first time that these inhibitory effects were mediated through direct binding of these two NRs to the IFN-γ promoter.

As previously discussed, RXR acts as both a homodimer as well as a heterodimer with PPARγ. Therefore, the next question is where and how these dimers associate with DNA to control IFN-γ transcription in HOZOT. The RXR homodimer interacts with a specific sequence, the DR-1 element that is found on a variety of promoters or enhancers of several distinct genes such as rat cellular retinol-binding protein type II, human apo-A-I, human apo-A-II, and rat growth hormone [25]. Interestingly, the DR-1 element is also located in the IFN-γ promoter region. Therefore, one possibility is that NEt3-IP exerts its inhibitory effects on IFN-γ production by forcing RXR homodimer to bind to IFN-γ's DR-1 element. Another possibility is that RXRα heterodimer binds to DNA and exerts its inhibitory effects, a phenomenon known as a permissive effect. In this case, heterodimer partners could be PPARγ or other NRs.

## 5. Conclusions

In conclusion, we found that RXRα and PPARγ were selectively expressed in HOZOTs and can be used as signature NRs. We also demonstrated that RXRα and PPARγ were functionally involved in cytokine production in HOZOT cells. Furthermore, both NRs repressed IFN-γ gene expression via binding to the PPRE/DR1 element in HOZOT cells. Our studies suggest a potential role for RXRα in the regulation of inflammation and might provide a pharmacological approach to modulating RXRα signaling as part of an overall strategy to suppress inflammation. Recently, Kakuta et al. [18] reported that type 2 diabetes was controlled in vivo by a treatment of NEt-3IP. Their observation supports the speculation of our study.

## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rinim.2012.08.001.

## References

[1] Laudet V. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. Journal of Molecular Endocrinology. 1997;19:207–226.
[2] Glass C.K., Saijo K. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. Nature Reviews Immunology. 2010;10:365–375.
[3] Wang K., Wan Y.J.Y. Nuclear receptors and inflammatory diseases. Experimental Biology and Medicine. 2008;233:496–506.
[4] Aggarwal S., Ghilardi N., Xie M.H., de Sauvage F.J., Gurney A.L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. Journal of Biological Chemistry. 2003;278:1910–1914.
[5] Komiyama Y., Nakae S., Matsuki T., Nambu A., Ishigame H., Kakuta S. et al. IL-17 plays important role in the development of experimental autoimmune encephalomyelitis. Journal of Immunology. 2006;177:566–573.
[6] Sato K., Suematsu A., Okamoto K., Yamaguchi A., Morishita Y., Kadono Y. et al. TH17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. Journal of Experimental Medicine. 2006;203:2673–2682.
[7] Ivanov I.I., McKenzie B.S., Zhou L., Tadokoro C.E., Lepelley A., Lafaille J.J. et al. The orphan nuclear recptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126:1121–1133.
[8] Benson M.J., Pino-Lagos K., Rosemblatt M., Noelle R.J. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. Journal of Experimental Medicine. 2007;204:1765–1774.
[9] Tang J., Zhou R., Luger D., Zhu W., Silver P.B., Grajewski R.S. et al. Calcitriol suppresses antiretinal autoimmunity through inhibitory effects on the Th17 effector response. Journal of Immunology. 2009;182:4624–4632.
[10] Nakamura S., Suzuki M., Sugimoto A., Tsuji-Takayama K., Yamamoto M., Otani T. et al. IL-2-independent generation of FOXP3$^+$CD4$^+$CD8$^+$CD25$^+$ cytotoxic regulatory T cell lines from human umbilical cord blood. Experimental Hematology. 2007;35:287–296.
[11] Suzuki M., Sugimoto A., Harashima A., Otani T., Yamamoto M., Nakamura S. et al. Novel mechanisms of suppressor activity exhibited by cytotoxic regulatory T cell lines, HOZOT. Experimental Hematology. 2009;37:92–100.
[12] Sugimoto A., Suzuki M., Otani T., Okochi A., Takeuchi M., Yamasaki F. et al. HOZOTs, novel human regulatory T-cell lines, exhibit helper or suppressor activities depending on dendritic cell or anti-CD3 stimulation. Experimental Hematology. 2009;37:1454–1463.
[13] Takeuchi M., Inoue T., Otani T., Yamasaki F., Nakamura S., Kibata M. Cell-in-cell structures formed between human cancer cell lines and the cytotoxic regulatory T-cell line HOZOT. Journal of Molecular Cell Biology. 2010;2:139–151.

[14] Tsuji-Takayama K., Suzuki M., Yamamoto M., Harashima A., Okochi A., Otani T. et al. IL-2 activation of STAT5 enhances production of IL-10 from human cytotoxic regulatory T cells, HOZOT. Experimental Hematology. 2008;36:181–192.
[15] Harashima A., Toraya T., Okochi A., Yamamoto M., Suzuki M., Otani T. et al. Interleukin-8 and RANTES are signature cytokines made by HOZOT, a new type of regulatory T cells. Molecular Immunology. 2009;46:3310–3319.
[16] Yamamoto M., Kondo E., Takeuchi M., Harashima A., Otani T., Tsuji-Takayama K. et al. miR-155, a modulator of FOXO3a protein expression, is underexpressed and cannot be upregulated by stimulation of HOZOT, a line of multifunctional Treg. PLOS One. 2011;6:e16841.
[17] Takamatsu K., Takano A., Yakushiji N., Morohashi K., Morishita K., Matsuura N. et al. The first potent subtype-selective retinoid X receptor (RXR) agonist possessing a 3-isopropoxy-4-isopropylphenylamino moiety, NEt-3IP (RXRα/β-dual agonist). ChemMedChem. 2008;3:780–787.
[18] Kakuta H., Ohsawa F., Yamada S., Makishima M., Tai A., Yasui H. et al. Feasibility of structual modification of retinoid X receptor agonists to separate blood glucose-lowering action from adverse effects: studies in KKA(y) type 2 diabetes model mice. Biological and Pharmaceutical Bulletin. 2012;35:629–633.
[19] Morishita K., Yakushiji N., Ohsawa F., Takamatsu K., Matsuura N., Makishima M. et al. Replacing alkyl sulfonamide with aromatic sulfonamide in sulfonamide-type RXR agonists favors switch towards antagonist activity. Bioorganic and Medicinal Chemistry Letters. 2009;19:1001–1003.
[20] Suzuki M., Yamamoto M., Sugimoto A., Nakamura S., Motoda R., Orita K. Delta-4 expression on a stromal cell line is augmented by interleukin-6 via STAT3 activation. Experimental Hematology. 2006;34:1143–1150.
[21] Xie C.Q., Jeong Y., Fu M., Bookout A.L., Garcia-Barrio M.T., Sun T. et al. Expression profiling of nuclear receptors in human and mouse embryonic stem cells. Molecular Endocrinology. 2009;23:724–733.
[22] Barish G.D., Downes M., Alaynick W.A., Yu R.T., Ocampo C.B., Bookout A.L. et al. A nuclear receptor atlas: macrophage activation. Molecular Endocrinology. 2005;19:2466–2477.
[23] Stephensen C.B., Borowsky A.D., KentLloyd K.C. Disruption of *Rxra* gene in thymocytes and T lymphocytes modestly alters lymphocyte frequencies, proliferation, survival and T helper type1/type2 balance. Immunology. 2007;121:484–498.
[24] Rasooly R., Schuster G.U., Gregg J.P., Xiao J.H., Chandraratana R.A.S., Stephensen C.B. Retinoid X receptor agonists increase Bcl2a1 expression and decrease apoptosis of naïve T lymphocytes. Journal of Immunology. 2005;175:7916–7929.
[25] Desvergne B. RXR: from partnership to leadership in metabolic regulations. Vitamins and Hormones. 2007;75:1–32.
[26] Faul M.M., Grese T.A. RXR modulators for the treatment of type II diabetes. Current Opinion in Drug Discovery. 2002;5:974–985.
[27] Nunez V., Alameda D., Rico D., Mota R., Gonzalo P., Cedenilla M. et al. Retinoid X receptor a controls innate inflammatory responses through the up-regulation of chemokine expression. Proceedings of the National Academy of Sciences of the United States of America. 2010;107:10626–10631.
[28] Clark R.B. The role of PPARs in inflammation and immunity. Journal of Leukocyte Biology. 2002;71:388–400.
[29] Malur A., Mccoy A.J., Arce S., Barna B.P., Kavuru M.S., Malur A.G. et al. Deletion of PPARγ in alveolar macrophages is associated with a Th-1 pulmonary inflammatory response. Journal of Immunology. 2009;182:5816–5822.
[30] Cunard R., Eto Y., Muljadi J.T., Glass C.K., Kelly C.J., Ricote M. Repression of IFN-gamma expression by peroxisome proliferator-activated receptor gamma. Journal of Immunology. 2004;172:7530–7536.
[31] Natarajan C., Bright J.J. Peroxisome proliferator-activated receptor-g agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. Genes and Immunity. 2002;3:59–70.
[32] Daynes R.A., Jones D.C. Emerging roles of PPARs in inflammation and immunity. Nature Reviews Immunology. 2002;2:748–759.